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Tumour-derived GM-CSF promotes granulocyte immunosuppression in mesothelioma patients

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23 **Conflict of Interest**

24 The authors declare that they have no potential conflict of interest.

25

Abstract

Purpose: The cross talk between tumour cells, myeloid cells, and T cells can play a critical role in tumour pathogenesis and response to immunotherapies. Although the aetiology of mesothelioma is well understood the impact of mesothelioma tumour cells on the surrounding immune microenvironment is less well studied. In this study the effect of the mesothelioma tumour microenvironment on circulating and infiltrating granulocytes and T cells is investigated.

Experimental Design: Tumour tissues and peripheral blood from mesothelioma patients were evaluated for presence of granulocytes, which were then tested for their T cell suppression potential. Different co-cultures of granulocytes and/or mesothelioma tumour cells and/or T cells were set up to identify the mechanism of T cell inhibition.

Results: Analysis of human tumours showed that the mesothelioma microenvironment is enriched in infiltrating granulocytes, which inhibit T cell proliferation and activation. Characterisation of the whole blood at diagnosis identified similar, circulating, immunosuppressive CD11b⁺CD15⁺HLADR⁻ granulocytes at increased frequency compared to healthy controls. Culture of healthy-donor granulocytes with human mesothelioma cells showed that GM-CSF upregulates NOX2 expression and the release of Reactive Oxygen Species (ROS) from granulocytes, resulting in T cell suppression. Immunohistochemistry and transcriptomic analysis revealed that a majority of mesothelioma tumours express GM-CSF and that higher GM-CSF expression correlated with clinical progression. Blockade of GM-CSF with neutralising antibody, or ROS inhibition, restored T cell proliferation suggesting that targeting of GM-CSF could be of therapeutic benefit in these patients.

49 **Conclusions:** Our study presents the mechanism behind the cross-talk between
50 mesothelioma tumours and the immune micro-environment and indicates that targeting
51 GM-CSF could be a novel treatment strategy to augment immunotherapy in patients with
52 mesothelioma.

53 **Translational Relevance**

54 The functional role of granulocytes and their cross talk with tumour cells and T cells in
55 human mesothelioma is not well understood. We demonstrated that GM-CSF is expressed by
56 mesothelioma tumour cells, and can polarize granulocytes to up regulate ROS production which
57 in turn suppresses the T cell proliferation and function. As GM-CSF plays a role in driving an
58 immunosuppressive granulocyte phenotype in mesothelioma, targeting GM-CSF could
59 represent an alternative therapeutic approach for these patients.

Introduction

Malignant mesothelioma is an aggressive cancer arising from the mesothelial cells lining the pleura, peritoneum and pericardium (1). The majority of patients present with advanced stage disease and are not candidates for surgery. Although chemotherapy improves outcome for these patients, the median overall survival is less than 24 months (2). Immunotherapy approaches relying on T cell anti-cancer activity, such as peptide vaccines and CAR T cells, have shown only limited efficacy suggesting that the underlying immune microenvironment may play a role in muting the immune response (3, 4).

Myeloid cells play an important role in the balance of pro- and anti- cancer T cell responses. Murine models of mesothelioma have shown that monocytes, macrophages, and dendritic cells may be modulated by the tumour microenvironment (5-7). However, the functional role of granulocytes and their mechanism of action in human mesothelioma is not well understood. Studies in mesothelioma have suggested the ratio between peripheral blood or intra-tumoural neutrophils and lymphocytes correlates with prognosis, indicating a key interaction between these cells in tumour pathogenesis (8). In other cancers, secreted factors within the tumour microenvironment control the differentiation of granulocytes. In turn this may promote inflammation within the tumour microenvironment or lead to changes in the interaction with the adaptive immune response. Here we investigate the mechanisms underlying the cross-talk between mesothelioma tumour cells, granulocytes and T cells.

Materials and Methods

Patients and sample collection

Heparinized blood samples were obtained from patients with malignant mesothelioma (n=47) who were enrolled in IRB approved protocols at the National Cancer Institute, Bethesda, USA and the University of Birmingham, UK before treatment (Table S1). Written informed consent was obtained from all the patients and the study was conducted in accordance with recognized ethical guidelines. Blood from healthy donors was obtained from the NIH Blood Bank (n=30) and at the University of Birmingham, UK (n=18), in heparin tubes. Patients with both histologically confirmed pleural (n=24) and peritoneal (n=9) mesothelioma were included in this study and at the time of enrolment had clinical and/or radiological evidence of disease. A number of patients had received prior treatments including surgery and systemic chemo or immune-therapy (Table S1). The transcriptomes of 87 mesothelioma tumours diagnosed between 1999 and 2013, held within the R2: Genomics Analysis and Visualisation Platform (<http://r2.amc.nl>) were analysed for CSF2 expression. Patients were aged from 28 to 81 years of age at diagnosis. 56 patients had a history of asbestos exposure, 14 had no history, and 17 were not known. Of the 87 patients' samples histologies were distributed as follows: 23 biphasic, 5 diffuse, 57 epithelioid, 2 sarcomatoid.

Cell lines

Human mesothelioma cell lines (ED(MSTO211)-H, AC-Meso Y9-Meso, MPM15, MPM26, MPM30, MPM34, MPM43) purchased from the Aichi Cancer Research Centre

Institute and Mesobank UK were cultured in RPMI-1640 (Invitrogen) with 10% heat-inactivated fetal bovine serum, glutamine (1X), sodium pyruvate (1X) and Penicillin-Streptomycin (RPMI 10%=R10%). The cell lines were cultured in a humidified atmosphere at 37°C with 5% CO₂. All cell lines were verified by Northgene (UK) DNA Short Tandem Repeat analysis within the last 6 months. All cell lines were tested of mycoplasma and were negative). Cell lines were used for up to 5 passages.

Flow cytometric analysis of whole blood and tumours

Whole blood and fresh tumour samples from diagnostic surgery were processed within 12h of collection. 10 samples from patients with benign pleural pathologies of infectious and inflammatory nature were also included as a comparison. Whole blood was either lysed using ammonium chloride solution according to manufacturer's instructions (Qiagen) or using a hypertonic ammonium chloride solution (150mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA) for 10 minutes at room temperature (maintained at 21-23°C) at a ratio of 1:9 (volume of sample: volume of lysing solution) prior to antibody staining. Where indicated peripheral blood was separated using a Lymphoprep density gradient. Tissue samples were digested using Type II collagenase (Worthington) for three hours at 37°C. Immune populations were identified by staining with anti- CD11b, anti-HLA-DR, anti-CD13, anti-CD14, anti-CD15, anti-CD66b, and anti CD45 antibodies (BD Biosciences) on ice or at room temperature for 30minutes. Cells were acquired using FACS-Canto II (BD Biosciences) and Cyan (Beckman Coulter) and analysed either by FCS Express 4 software (DeNovo Software) or FlowJo (Tree Star).

126

127 **Isolation of granulocytes, T cells, and mesothelioma cells for functional assays**

128 The whole blood from healthy donors and patients were processed as described above.
 129 We isolated the low density granulocytes from the peripheral blood mononuclear layer
 130 and high density granulocytes from the layer of white cells on the red cell pellet,
 131 following Lymphoprep centrifugation, by magnetic bead isolation using anti-CD15
 132 microbeads (BD Pharmingen) and MACS LS separation columns (Miltenyi Biotech)
 133 according to manufacturer's instructions. Cell purity was >98% as confirmed by flow
 134 cytometry. Cell populations were similarly isolated from collagenase digested tumours
 135 using MACS beads (anti-CD15 for granulocytes and anti-CD14 for monocytes), followed
 136 by flow cytometric confirmation of purity. The dose of collagenase selected has
 137 previously been established to not affect cell surface marker expression or cell viability.

138 For isolation of autologous T cells and myeloid cells from the whole blood the target
 139 populations were enriched first using positive selection with CD45 magnetic beads
 140 (Miltenyi Biotech), followed by staining with myeloid antibodies (above) and anti-CD3
 141 antibody (Biolegend). Cells were sorted on Astrios (Beckman Coulter) using a 100µm
 142 nozzle. DAPI was used as a viability marker to gate out the dead cells.

143

144 **Granulocyte polarisation**

145 To generate tumour conditioned media (TCM), cell lines or sorted patients' tumour cells
 146 were plated (1.5×10^6 cells) and cultured for 72 hours. The conditioned media was
 147 removed and filtered prior to use. Following lymphoprep isolation high density

granulocytes were enriched by CD15 magnetic bead isolation as above, healthy donor granulocytes were plated in R10% in 24 well plates, at concentrations of 1×10^6 per well. TCM was added as 25% of the total volume as indicated. Granulocytes were harvested following 24 hours of culture, washed twice prior to use in suppression assays. Granulocyte viability was confirmed to be >90% in all cases, by flow cytometry, before further experimentation.

Autologous T-cell proliferation assays

Sorted $CD3^+$ T cells were labelled with $10 \mu M$ carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies) and cultured with sorted granulocytes at ratios of 1:0, 1:0.5, 1:1 in complete media at $37^\circ C$, 5% CO_2 for 4 days in the presence of 1:1 ratio of anti-CD3/ anti-CD28 dynabeads (Invitrogen). Cells were stained with V450 anti-CD4 (Clone-RPA-T4; BD Biosciences) and APC-Cy7 anti-CD8 (Clone-RPA-T8; BioLegend) and proliferation was determined by CFSE dilution. Unstimulated T cells were used as a negative control. The effect of the addition of L-NMMA (0.5mM, NG-Methyl-L-arginine acetate), nor-NOHA (0.5mM, N-Omega-hydroxy-nor-L-arginine) and iNAC (10mM) (all from Sigma Aldrich) was similarly tested. The percentage of cells that diluted CFSE (divided cells) was determined.

Peripheral Blood Lymphocyte cell proliferation assay

Peripheral blood lymphocytes (PBLs) (2×10^5) were cultured in 96 well flat bottom plates with coated anti-CD3 antibody ($3 \mu g/mL$) and anti-CD28 antibody ($2 \mu g/mL$), in

200μL R10%. Cells were incubated at 37°C, 5% CO₂ for 4 days and then 1μCi/well ³H-thymidine (Perkin Elmer Life Sciences) was added for 12-16 hours. ³H-thymidine incorporation was measured using a TopCount reader (Perkin Elmer). The suppressive ability of autologous or conditioned granulocytes was assessed by co-culturing purified cells together with the PBLs. nor-NOHA (0.5mM), L-NMMA (0.5mM), iNAC (10mM; Sigma Aldrich) was added to cells in culture. 25mM HEPES was added to the medium to maintain the pH after iNAC addition. Data are expressed as a percentage of PBL proliferation driven by antibody co-stimulation in the presence of MDSC, compared with PBL proliferation in the absence of suppressive cells (100%).

Reactive Oxygen Species assay

Sorted granulocytes were stained with 2', 7'- dichlorofluorescein diacetate (DCFDA) using DCFDA cellular ROS detection assay kit (Abcam) for 30 minutes at 37°C. The stained cells were analyzed on a BD FACS Calibur and Cyan (Beckman Coulter). Cells stained with Tert-butyl hydrogen peroxide (TBHP), TCM polarized granulocytes were also incubated with Phorbol 12-myristate 13-acetate (PMA) (concentration need to be added) during the staining with DCFDA, this was used as a positive control.

Quantification of H₂O₂ production was measured using the Amplex Red Hydrogen Peroxidase assay kit (Invitrogen). Following culture in mesothelioma conditioned media for 24 hours, sorted granulocytes were washed twice in R10%, counted and plated in Krebs–Ringer phosphate buffer, according to manufacturer's guidelines. Detection of

H₂O₂ was carried out following 30 minutes of incubation at 37°C using a microplate reader at 560nm.

ELISA

The concentrations of cytokines within conditioned media following culture with T cells, mesothelioma cell lines (1x10⁶/mL) or sorted tumour cells were quantified using a competitive enzyme linked immunoassay according to the manufacturers' instructions. The following molecules were tested GM-CSF (Biolegend), IL-13 (BD Biosciences), IL-8 (Biolegend), IL-6 (Biolegend), G-CSF (R&D Systems), VEGF (R&D Systems), Mesothelin (Biolegend). The concentration of IFN-γ in co-culture supernatants was determined by Ready Set Go ELISA kit (eBioscience).

RT-Q-PCR analysis

RT-Q-PCR was used to detect NOX2 expression in cell line supernatant conditioned granulocytes (0, 4, 8, 12, 24 hour time points). RNA was extracted using an RNeasy Mini kit (Qiagen). cDNA was prepared using SuperScriptTM III Reverse Transcriptase (Invitrogen, CA) following the manufacturer's instructions. RT-Q-PCR was done in duplicate using FAST SYBR Green Master Mix (Applied Biosystems) and the Applied Biosystems 7500 Fast Real-Time PCR system. Analysis of gene expression was calculated according to 2^{-ΔT} method and plotted as arbitrary units of mRNA relative to GAPDH. Gene specific primer sequences were NOX2 (CAAGATGCGTGGAACATA,

F; TCCCTGCTCCCACTAACA, R) and GAPDH (CCAGCCGAGCCACATCGCTC, F;
 ATGAGCCCCAGCCTTCTC, R) (Eurofins).

Immunohistochemistry

Mesothelioma sections, from diagnostic tumour biopsies (n=38), were deparaffinised in
 HistoClear (National diagnostics) and ethanol, and rehydrated in 0.3% hydrogen peroxide
 for 15 minutes. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0)
 for 20 minutes in a microwave oven. Slides were cooled and washed prior to blocking in
 5X Caesin (Thermofisher) for 30 minutes at room temperature. Sections were then
 incubated over night with primary antibody, rabbit anti-GM-CSF (Novus Biologicals),
 diluted in PBS. Sections were washed and secondary antibody (Universal ImmPRESS
 antibody, Vector Laboratories) was added at room temperature for 30 minutes, followed
 by further washing and addition of DAB substrate (ImmPACT DAB, Vector
 Laboratories) for 5 minutes. After counterstaining with Harris haematoxylin (Sigma),
 slides were dehydrated using ethanol and HistoClear and mounted using Omnimount
 (National diagnostics). Slides were examined and photographed using a Nikon Eclipse
 400 microscope.

Statistical analysis

Continuous parameter values were compared between two groups using an exact form of
 a Wilcoxon rank sum test. Paired comparisons were performed using a Wilcoxon signed
 rank test. Spearman correlation analysis was used to determine the correlation between

age and MDSC parameters. The correlations are interpreted as follow: strong if $|r| > 0.70$; moderately strong if $0.50 < |r| < 0.70$; weak to moderately strong if $0.30 < |r| < 0.50$; weak if $|r| < 0.30$. All p-values are two-tailed and presented without adjustment for multiple comparisons because all tests performed were considered to be exploratory.

Results

Mesothelioma tumours modulate infiltrating myeloid cells to suppress T cell responses

The immune microenvironment in mesothelioma has been shown to have strong prognostic implications, with infiltration by CD8⁺ lymphocytes conferring a favourable prognosis (9) and the association of peripheral blood granulocyte –to-lymphocyte ratio with poorer prognosis (10-12). However our understanding of the biological cross-talk between mesothelioma cells, granulocytes, and T cells in human patients is limited.

Interrogation of the transcriptomic profile of 87 mesothelioma tumours, held within the R2: Genomics Analysis and Visualisation Platform (<http://r2.amc.nl>), suggested a significant infiltration of immune cells in the tumour microenvironment (Fig. 1a). Flow cytometric analysis of 18 digested, human mesothelioma tumours confirmed this data at the cellular level within the tumour microenvironment (Mean: CD15= 8.6%, CD14= 4.8%, CD3=6.7%; Fig. 1b). Immuno-histochemical staining of a further 38 mesothelioma tumours supported the findings, with identification of granulocytes in all samples at diagnosis (Figure 1c). Characterisation of tumour-associated granulocytes by flow-cytometry showed that they expressed CD11b, CD15, with low/absent CD33 expression,

and low/absent HLA-DR expression (Fig. 1d). Murine model of mesothelioma recently identified that the immune-regulatory transcriptome of granulocytes may be altered within the tumour microenvironment with potential effects on surrounding T cells and tumour cells.(13) To investigate the effects of granulocytes on T cells, CD15⁺ granulocytes were sorted from mesothelioma tumours at the time of resection, and co-cultured with T cells from healthy donors. Tumour derived granulocytes suppressed T cell proliferation to a greater extent, compared to those from healthy donor blood or those extracted from pleural tissue with benign pathologies (Fig. 1e). Similarly CD14⁺ monocytes from mesothelioma tumours were sorted and also found to suppress T cell proliferation (p=0.0002) (Supplementary Fig. 1a). Therefore the tumour microenvironment is able to locally modulate infiltrating myeloid cells to inhibit T cell proliferation.

Mesothelioma creates a systemic immunosuppressive environment through circulating granulocytes

The effects of mesothelioma tumours on the immune system may be limited to the local tissue microenvironment or could also lead to systemic alteration. To test this we compared T cells from the blood of patients at diagnosis to those from healthy donors, and observed that mesothelioma patients' T cells have a reduced proliferation capacity compared to those in healthy donors (Fig. 2a). We have previously identified that tumour-metabolism of arginine can create a systemic environment inhibitory to T cell responses. As mesotheliomas are known to be arginine auxotrophs, we measured the arginase activity of mesothelioma cell lines (14). No significant arginase activity was

identified suggesting an alternative mechanism must be responsible (Supplementary Fig. 1b). As we identified immunosuppressive granulocytes infiltrating mesothelioma tumours, we therefore hypothesised the T cell suppression may be due to the presence of these circulating immunosuppressive myeloid cells.

To investigate the hypothesis, the frequency of granulocytic and monocytic cells was characterised in the whole blood of healthy donors and mesothelioma patients (n=33) at diagnosis (Table S2). There were significant increases in the percentage of HLA-DR⁻ granulocytes (CD14⁻CD15⁺CD11b⁺HLADR⁻) in the whole blood, compared to healthy controls (p=0.013) (Fig. 2b). Subpopulation analysis revealed only a marginal difference in HLA-DR⁻ monocytes (CD14⁺CD15⁻CD11b⁺HLADR⁻) compared to healthy donors (p=0.05) (Figure 2c) and no difference in the frequency of HLA-DR⁺ monocytes (CD14⁺CD11b⁺HLADR⁺) (median 7.0% versus 8.9%; p=0.14) or HLA-DR⁺ granulocytes (CD15⁺CD14⁻CD11b⁺HLADR⁺) cells (median 0.83% vs. 1.46%; p=0.29) between healthy donors and mesothelioma patients. Consistent with reports of increased granulocyte:lymphocyte ratios in mesothelioma patients, granulocytes were the most predominant population overall (Median Frequency: CD14⁺ Monocytes⁻: 0.198% vs CD15⁺ Granulocytes⁻: 66.8%). Immunophenotyping revealed significant differences in the relative expressions of granulocyte markers CD11b and CD66b compared to those from healthy donors (Fig. 2d and Supplementary Fig. 1c).

As CD15⁺ granulocytes are the major population of circulating myeloid cells in mesothelioma patients and shared the same immunophenotype as tumour-infiltrating granulocytes described above, their functional effects on T cells was examined further. Co-culture of sorted whole blood granulocytes from patients decreased both autologous

CD4⁺ and CD8⁺ T cell proliferation at ratios of 1:1 and 1:0.5 (T cells:granulocytes) (Fig. 2e and Supplementary Fig. 1d) and activation (p=0.0078, Fig. 2f).

Immunosuppressive granulocytes may be methodologically identified in the PBMC layer (classical G-MDSC; low-density) and on the red cell pellet (high-density) following density gradient centrifugation of whole blood. Analysis of separated whole blood identified a significant increase in the frequency of granulocytes within the PBMC layer, however the frequency is extremely low (median <10%) with 90% of the granulocytes lying on the red cell pellet (Figure 3a). This small population of granulocytes had only a weak ability to suppress T cell proliferation (Fig. 3b). Only the high-density granulocytes, which had increased frequency in the patients, had suppressive activity (Figure 3b). Granulocytes from healthy donors had minimal effects on T cell proliferation (Fig. 1e and Supplementary Fig. 2a) or IFN- γ release (Supplementary Fig. 2b). Therefore granulocytes within the blood and tumours of mesothelioma patients share the same immunophenotype and functional capacity to suppress T cell proliferation and activation, thus extending the immunosuppressive microenvironment.

Mesothelioma conditioned granulocytes suppress T cell proliferation by generation of reactive oxygen species

Granulocytes can impair T cell proliferation through a number of mechanisms including arginine depletion, nitric oxide species or reactive oxygen species production, and release of immunosuppressive cytokines (15-19). We examined iNOS and Arginase I expression in patients' granulocytes and those from healthy donors identifying no significant

differences in expression (Fig.3c). The addition of the Arginase or iNOS inhibitors, nor-
 NOHA or L-NMMA respectively, to sorted patients' granulocytes did not rescue T cell
 proliferation thus excluding these mechanisms. (Fig.3d). No evidence for
 immunosuppressive cytokine release from these cells was identified in patient plasma by
 ELISA (IL-10, IL-1 β , IL-4, IL-13 Supplementary 2c). In a mesothelioma murine model
 Reactive Oxygen Species have been demonstrated to suppress T cell responses (20).
 Gating on granulocytes identified that mesothelioma patients' upregulate ROS, compared
 to healthy controls ($p=0.03$; Fig. 3e, Supplementary Fig. 2d). Addition of the ROS
 inhibitor iNAC, to sorted patients' granulocytes restored both autologous CD4⁺ and CD8⁺
 T cells proliferation (Fig. 3f and Supplementary Fig. 2e) and IFN- γ release (Fig. 3g). We
 confirmed that ROS production was reduced by the addition the inhibitor iNAC
 ($p=0.031$, Fig. 3h). PDL1 is another mechanism that myeloid cells may use to modulate T
 cells. There was no significant difference in the frequency of CD15⁺PDL1⁺ cells in the
 blood or tumours of patients compared to those from healthy controls (Supplementary
 Fig. 2f). Correlating the frequency of PDL1⁺CD15⁺ cells with CD3⁺ frequency revealed
 no significant correlation in the blood ($p=0.4976$, $r=-0.3214$) (Supplementary Figure 2f),
 but there was a significant correlation in the tumour ($p=0.0583$, $r=0.8286$)
 (Supplementary Fig 2g and h). The findings suggest that in the tumour, granulocyte
 PDL1 may be a secondary mechanism of modulating T cell numbers inside the tumour
 microenvironment, but not peripherally.

GM-CSF from mesothelioma tumour cells drives granulocyte ROS production

Although it is recognised that mesothelioma tumours release G-CSF, which may contribute to granulocyte expansion and recruitment, the mechanism by which granulocytes are polarised to upregulate ROS production and suppress T cells is unknown (21, 22). Granulocytes produce reactive oxygen species through the activity of NADPH oxidase enzyme (NOX2) expression. Consistent with this we demonstrated NOX2 is expressed in patients' granulocytes and healthy donors (Fig. 4a). To examine the effect of the mesothelioma microenvironment on granulocytes, healthy-donor derived granulocytes were cultured in the conditioned supernatants of mesothelioma cell lines or primary tumours. Conditioned supernatants led to an upregulation of NOX2 expression over time (Fig. 4b), with accompanying increase in the production (Figure 4c, Supplementary Fig. 3a and 3b) and release of reactive oxygen species (Fig. 4d). PMA was used as positive control for ROS induction in granulocytes upregulation, confirming the mesothelioma specific mechanism (Supplementary Fig. 3c). The mesothelioma-conditioned granulocytes showed a strong ability to suppress T cell proliferation (Figure 4e), which could be rescued by the addition of iNAC (Fig. 4f). LOX-1 has been reported to be a marker for some granulocytic MDSCs (23). Conditioned media led to no change in LOX-1 on healthy donor granulocytes (Supplementary 3d). Therefore mesothelioma cells signal to granulocytes to modulate their function.

To identify the nature of the mesothelioma-granulocyte cross-talk, mesothelioma conditioned supernatant was first boiled to denature all proteins. Boiled supernatant lost the ability to polarise granulocytes to suppress T cell proliferation (Supplementary Figure 3e) consistent with the release of a soluble molecule from the mesothelioma cells. Arginine depletion, a potential mechanism of polarisation due to mesothelioma arginine

auxotrophism, similarly did not polarize healthy donor granulocytes to produce ROS consistent with this finding (Supplementary 3f).

ELISAs for cytokines involved in granulocyte signalling were performed of supernatants from mesothelioma cell lines and mesothelioma human primary tumour cells, identified a number of key molecules were highly expressed – G-CSF, GM-CSF, IL-13, IL-6, IL-8, VEGF, PGE2, Mesothelin, (Fig. 5a). In particular mesothelioma cells release IL-8 (neutrophil chemotactic protein; mean concentration 981pg/ml) and G-CSF (mean concentration 283pg/ml) which are known to attract granulocytes into the tumour microenvironment. No evidence of serum amyloid-A release from mesothelioma cells was found. Interrogation of the R2 database confirmed a similar cytokine expression profile from 87 human tumours (Fig. 5b). Culture of healthy-donor granulocytes with individual recombinant cytokines identified above, showed that GM-CSF led to the highest upregulation of ROS production (Fig. 5c) with associated upregulation in NOX2 expression (Supplementary Fig. 3g). Granulocytes treated with recombinant GM-CSF inhibited T cell proliferation (Fig. 5d and 5e), and T cell proliferation was rescued by the inhibitor (iNAC) or removal of reactive oxygen species with catalase (Fig. 5e). No increases in plasma GM-CSF concentrations are identified in patients at diagnosis, suggesting that the intra-tumoural release of GM-CSF drives the ROS upregulation in granulocytes. (Supplementary Fig. 4a) Addition of anti-GM-CSF neutralising antibody to mesothelioma cell line co-cultures inhibited ROS upregulation (Fig. 6a and 6b) and the release of peroxide species in conditioned granulocyte supernatants (Supplementary Fig 4b) confirming mesothelioma-released GMCSF drives granulocyte ROS production and T cell suppressive activity. The addition of anti-GM-CSF neutralising antibody rescued T

cell proliferation, confirming the mechanism of mesothelioma polarisation of granulocytes (Fig. 6c).

ROS production from conditioned healthy granulocytes correlated with the concentration of GM-CSF in tumour conditioned media ($r=0.438$, $p=0.0118$) (Fig. 6d). Immunohistochemistry of mesothelioma tumours confirmed that GM-CSF is expressed within the tumour microenvironment of patients (Fig. 6e and Supplementary Fig. 4c) and transcriptomic analysis of 87 primary tumour samples within the R2: database demonstrated that GM-CSF is expressed in over 50 % of the samples, and does not correlate with histological subtype (Fig. 6f).

In summary mesothelioma creates an immunosuppressive microenvironment locally and systemically through the release of GM-CSF from tumour cells which induces granulocyte ROS production to inhibit T cell function.

Discussion

Although the role of monocytes and macrophages in mesotheliomas has previously been well documented in human tissue and murine models, granulocytes have received little attention. In this study we focused on human mesotheliomas, identifying the mechanism by which tumour cells modulate granulocyte function to suppress T cell responses. A previous immune-histochemical study identified that high CD4 T cell counts or low neutrophil counts within mesothelioma tumours are linked to better patient outcomes (24). To evaluate the seemingly reciprocal relationship between granulocytes and T lymphocytes we first confirmed that granulocytes make up a significant proportion of

414 infiltrating immune cells, with relatively fewer T cells. To date granulocyte function in
 415 mesotheliomas has almost exclusively been studied in murine cell line xenografts.
 416 Murine granulocytes may be alternatively activated in mesotheliomas (N1 vs N2) or
 417 characterised as granulocytic MDSCs (G-MDSC) (13, 20, 25). In all of these murine
 418 cases the granulocytic cells express reactive oxide species – a well-established
 419 mechanism of T cell suppression (26). We carefully considered whether our tumour-
 420 infiltrating and circulating granulocytes could be G-MDSC according to recent guidelines
 421 for nomenclature which define MDSCs based on immunophenotype, density, and
 422 suppressive activity (27). In our mesothelioma patients both circulating and tumour-
 423 associated granulocytes were CD11b⁺CD14⁺CD15⁺/CD66b⁺, fitting with the G-MDSC
 424 phenotype. However blood G-MDSCs are classically described as being low density
 425 cells, following separation with density centrifugation. We showed that in mesothelioma
 426 patients' blood, low density granulocytes are a minority population within the PBMC
 427 layer and have minimal T cell suppressive activity – thus the two populations are distinct.
 428 In addition no evidence of altered LOX-1 expression, a marker recently identified on low
 429 density G-MDSCs, was found after mesothelioma conditioning of healthy donor
 430 granulocytes (23). The most suppressive granulocytes are those of high density, and act
 431 through ROS release. The intra-tumoural granulocytes we studied share the same
 432 immunophenotype and suppressive mechanism. As discussed in the consensus
 433 recommendations based on current technology there is no unique marker to distinguish
 434 suppressive granulocytes from G-MDSC, particularly for intra-tumoural cells. Notably
 435 the need to use cell density on separation as a method to define immune cell subsets is
 436 extremely limited, and alternative methodologies will be developed for the future

characterisation of these cells. Our findings highlight the plasticity of granulocytes in humans and their place in regulating the tumour associated immune microenvironment (13). Similar examples of human cancer-associated, immunosuppressive granulocytes, as opposed to G-MDSC, have been identified in melanoma and non-small lung cancer, driven through the release of tumour derived factors (15, 28).

Our analysis of the supernatants of mesothelioma cell lines and primary tumor tissue revealed a cytokine profile consistent with granulocyte attraction and modulation within the tumor microenvironment. A number of factors have been reported to modulate granulocyte function in murine models of mesothelioma. In a murine model of mesothelioma, prostaglandin inhibition reduced the number of granulocytic MDSCs (20). TGF-beta within murine mesothelioma tumors also drives the expression of the chemokines CCL3, CCL5, and CCL2 in pro-tumoral granulocytes (29). For humans no direct mechanism of mesothelioma modulation of granulocytes has been shown although the mesothelioma inducing mineral erionite can directly stimulate ROS production in healthy donor-derived neutrophils (30). IL-8 (CXCL8) is a potent pro-inflammatory cytokine and is primarily known for its chemotactic and activating action on neutrophils, along with inhibition of normal neutrophil apoptosis (31-33). Our finding of moderate levels of IL-8 released from mesothelioma cells, may contribute to the enhanced granulocytes infiltration of mesothelioma tumours.. Targeting of IL-8 in models of tumours such as fibrosarcoma and prostate carcinoma prevents the influx of host neutrophils (34). IL-8 is also an autocrine growth factor in a number of cancer types (35-37), including mesothelioma (38).

We identified that mesotheliomas can also release G-CSF, a second well established cytokine that induces granulocyte infiltration. Notably G-CSF production by mesothelioma is reported to confer a more aggressive phenotype (39-41). Although we confirmed mesothelioma tumours release IL-8 and G-CSF or prostaglandins, these factors had no impact in generating suppressive granulocytes. Instead we demonstrated that GM-CSF is expressed by mesothelioma tumor cells, and can polarize granulocytes to upregulate ROS production. No differences in the effect of granulocyte derived ROS was found on CD4+ versus CD8+ T cells. Establishment of cell lines from primary mesotheliomas have reported significant production of GM-CSF (42) and this cytokine can drive suppressive granulocyte activity in murine models for a number of solid tumors (43-45). Although we identified GM-CSF was widely expressed in our samples studied, the effects of prior therapies in our patient population, on GM-CSF expression is unknown.

Clinically GM-CSF has been used as an alternative to G-CSF to support myeloid cell recovery post-chemotherapy in mesothelioma patients (46, 47). No differences in outcome were reported for the two growth factors, although the effects on immune parameters are not available. Recombinant GM-CSF has also been administered alongside a tumor vaccine in this patient group (48-50) and used alongside immunotherapy approaches in neuroblastoma. In the mesothelioma studies GM-CSF was administered to patients in all study arms, regardless of whether they received the investigational tumour/peptide vaccines or not. Although responses are noted, it is not possible to understand whether the cytokine had any effect on outcomes both within the trial populations or compared to historical controls. It is possible that administration of

GM-CSF may inhibit anti-tumor T cell responses, through the induction of G-MDSC, contributing to the lack of clinically relevant T cell responses seen in these patients. In two trials where GM-CSF was administered intra-lesionally to mesothelioma, neutrophil infiltration and maturation was enhanced, however, this was not associated with tumor responses in the majority of patients (51, 52). Indeed a Phase II clinical trial in neuroblastoma demonstrated difference in prognosis if GM-CSF is administered intravenously vs subcutaneously, which could impact the dose-dependent effects of this cytokine on granulocyte phenotype (53, 54).

Preclinical studies which block GM-CSF have resulted in reversal of T cell inhibition by MDSCs in the setting of pancreatic tumours, and improvements in phenotype in inflammatory disease models (55, 56). Our data suggests that targeting the GM-CSF pathway may be of benefit in mesothelioma. Clinically relevant approaches to target GM-CSF have been focused on inflammatory diseases (57). Mavrilimumab (CAM-3001) is a human anti-GM-CSF receptor- α antibody which has completed Phase I and II clinical trials in the setting of rheumatoid arthritis (58). Our findings suggest that anti-GM-CSF or anti-GM-CSF receptor antibodies could play a critical role in mesothelioma treatment, particularly alongside T cell immunotherapies.

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508

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Figure Legends

Figure 1. Granulocytes in tumor tissue suppress T cell proliferation.

a) Transcriptomic expression of CD14, CD15, CD3E, and Mesothelin in 87 mesothelioma tumours from the R2: Genomics Analysis and Visualisation Platform (<http://r2.amc.nl>).

b) Percentages of CD15⁺, CD14⁺, and CD3⁺ cells detected by flow cytometry in the digested tumor tissue of 18 patients with confirmed mesothelioma.

c) Representative tumor sections from 6 patients (total stained = 38 tumours) demonstrating infiltration of CD15 expressing cells within mesothelioma tumours. Images were taken at 20X magnification.

d) Immunophenotype of tumour-associated granulocytes by flow-cytometry identified they expressed CD11b, CD15, with low/absent CD33 expression, and low/absent HLA-DR expression.

e) T cell proliferation from healthy donors is suppressed following culture with CD15⁺ granulocytes (representative 1:0.5 ratio) sorted from mesothelioma tumours, compared to those cultured in complete media alone, with CD15⁺ granulocytes from the blood of healthy donors, or from pleural tissue with benign pathologies.

Figure 2. Granulocytes are elevated in peripheral blood of mesothelioma patients

and suppress T cell proliferation and activation a) Sorted CD3⁺ T cells from the blood

of mesothelioma patients (6 untreated, 2 with prior therapy) have reduced proliferative capacity compared to those sorted from the blood of healthy donors.

b) Increased frequency of CD15⁺ granulocytes in the peripheral blood of mesothelioma patients (n=33) and healthy donors (n=30) at diagnosis.

c) Marginal increased frequency of CD14⁺HLADR⁻ monocytes in the peripheral blood of mesothelioma patients (n=33) and

healthy donors (n=30). d) Expression of CD15, CD16, CD11b, and CD66b markers on granulocytes of healthy donors and mesothelioma patients, as detected by flow cytometry. e) Autologous CD4⁺ and CD8⁺ T cell proliferation is suppressed following culture with CD15⁺ granulocytes sorted from the blood of patients at diagnosis. T cells and granulocytes were co-cultured at ratios of 1:0.5 and 1:1 respectively and compared to T cells alone (1:0). f) T cell-derived IFN- γ release in culture supernatants is significantly impaired following co-culture with CD15⁺ granulocytes sorted from the blood of patients at diagnosis.

Figure 3. Granulocytes from mesothelioma patients suppress T cell proliferation

through ROS a) The frequency of CD11b⁺CD15⁺ cells was compared in the whole blood and PBMC layer following Lymphoprep separation, for 18 mesothelioma patients and 12 healthy donors. The majority of CD15⁺ granulocytes lie on the red cell pellet following lymphoprep separation. b) Healthy donor T cell proliferation is most suppressed following culture in the presence of blood CD15⁺ granulocytes from mesothelioma patients which have been collected from the red cell pellet (High Density) after Lymphoprep separation. Low density granulocytes isolated in the PBMC layer of the same blood samples were comparatively less suppressive to T cell proliferation. c) QPCR analysis of the expression of iNOS and Arginase in granulocytes sorted from healthy donors or patients d) T cell proliferation is not restored by the addition of L-NMMA or nor-NOHA to the cultures in the presence of CD15⁺ granulocytes from patients. 2 representative patients are shown. e) Increased frequency of ROS⁺ CD15⁺ granulocytes in the blood of patients from mesothelioma patients compared to healthy donors f)

Inhibition of NOX2 activity with iNAC reversed the suppressive effect of granulocytes on CD4 and CD8 T cell proliferation. g) Inhibition of NOX2 activity with iNAC restored T cell activation, as measured by IFN- γ release into cell supernatants. h) Culture of patients' granulocytes with iNAC reduced the intracellular production of ROS confirming the known specificity of drug action.

Figure 4. ROS generation by granulocytes is upregulated by the mesothelioma microenvironment a) Expression of NOX2 by qRT-PCR in CD15⁺ cells from the blood of healthy donors and mesothelioma patients. b) NOX2 expression in CD15⁺ granulocytes is upregulated over time following co-culture with mesothelioma cell lines, as assessed by qRT-PCR. c) ROS production is up-regulated in healthy-donor derived granulocytes following culture in conditioned media from sorted mesothelioma malignant cells or mesothelioma cell lines, compared to complete RPMI. ROS species are detected by DCFDA staining and flow cytometry. d) Release of ROS from CD15⁺ granulocytes is upregulated after culture with mesothelioma cell lines or sorted mesothelioma malignant cells as detected by hydrogen peroxide species, using a colorimetric assay. e) T cell proliferation is significantly inhibited following culture with cell line conditioned- or tumour conditioned- granulocytes. Comparison made with T cells cultured with granulocytes conditioned by completed media alone. f) Treatment of mesothelioma cell line conditioned granulocytes with iNAC prevents suppression of T cell proliferation

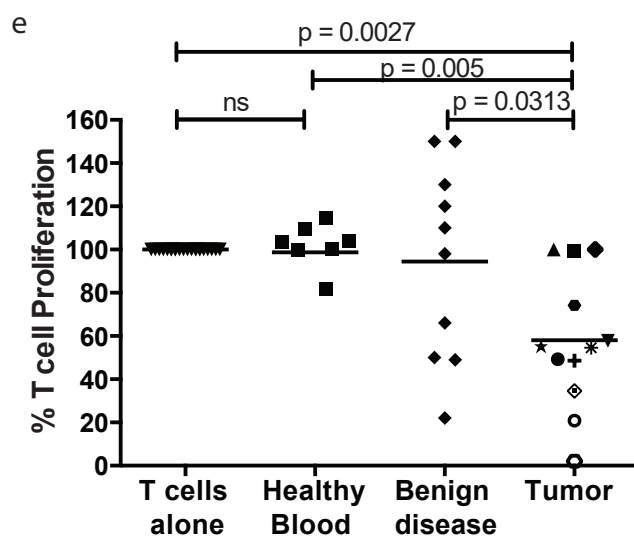
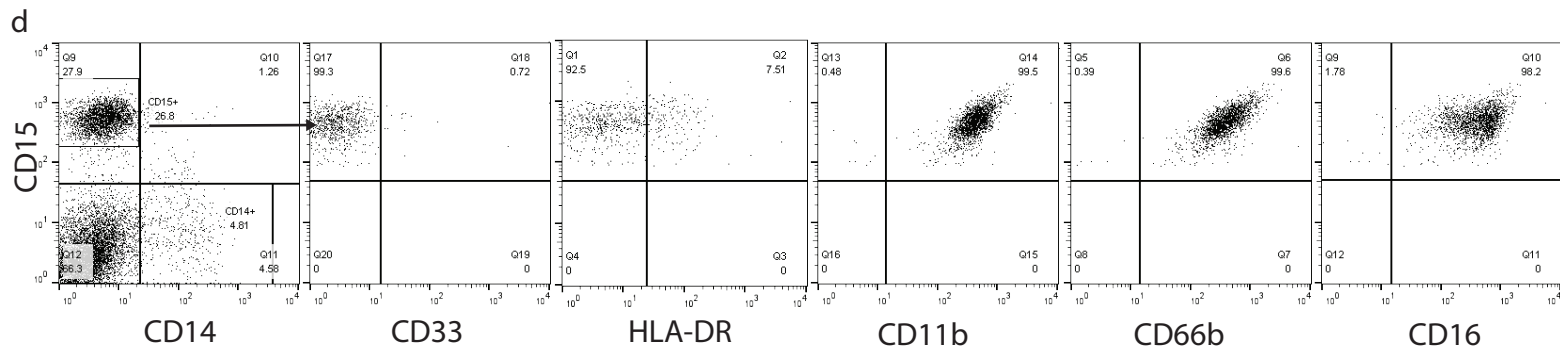
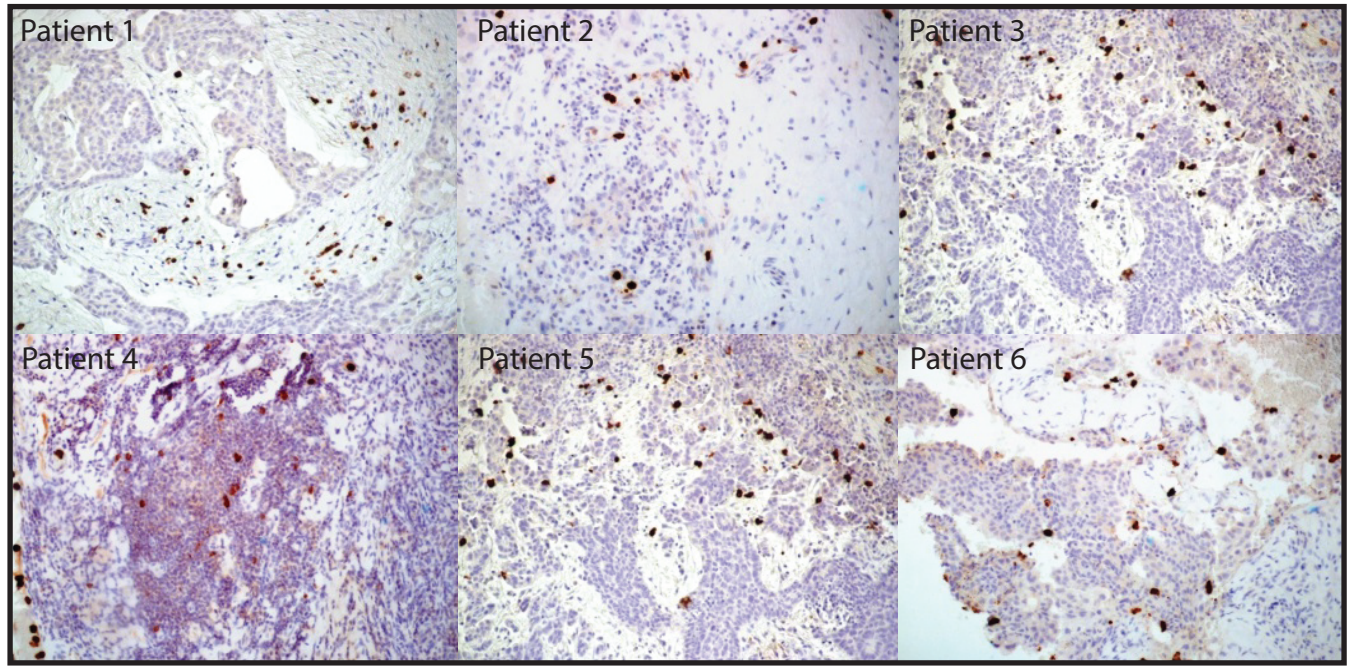
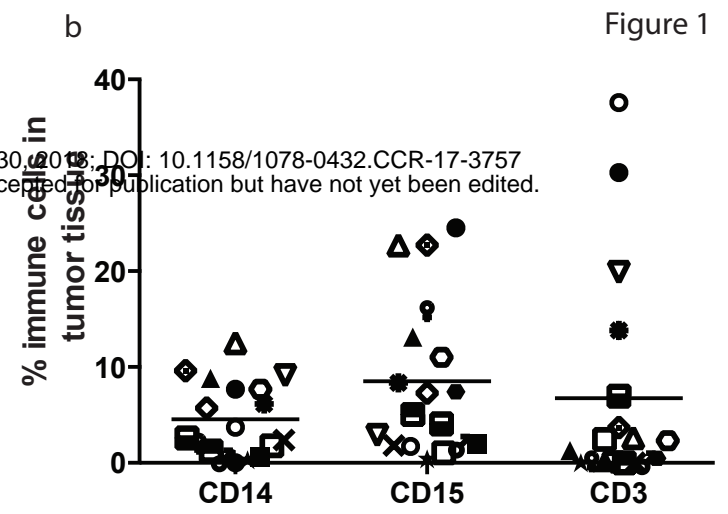
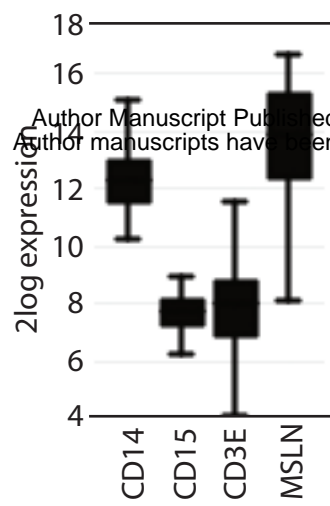
Figure 5: Mesothelioma cells release GM-CSF to up-regulate granulocyte ROS and

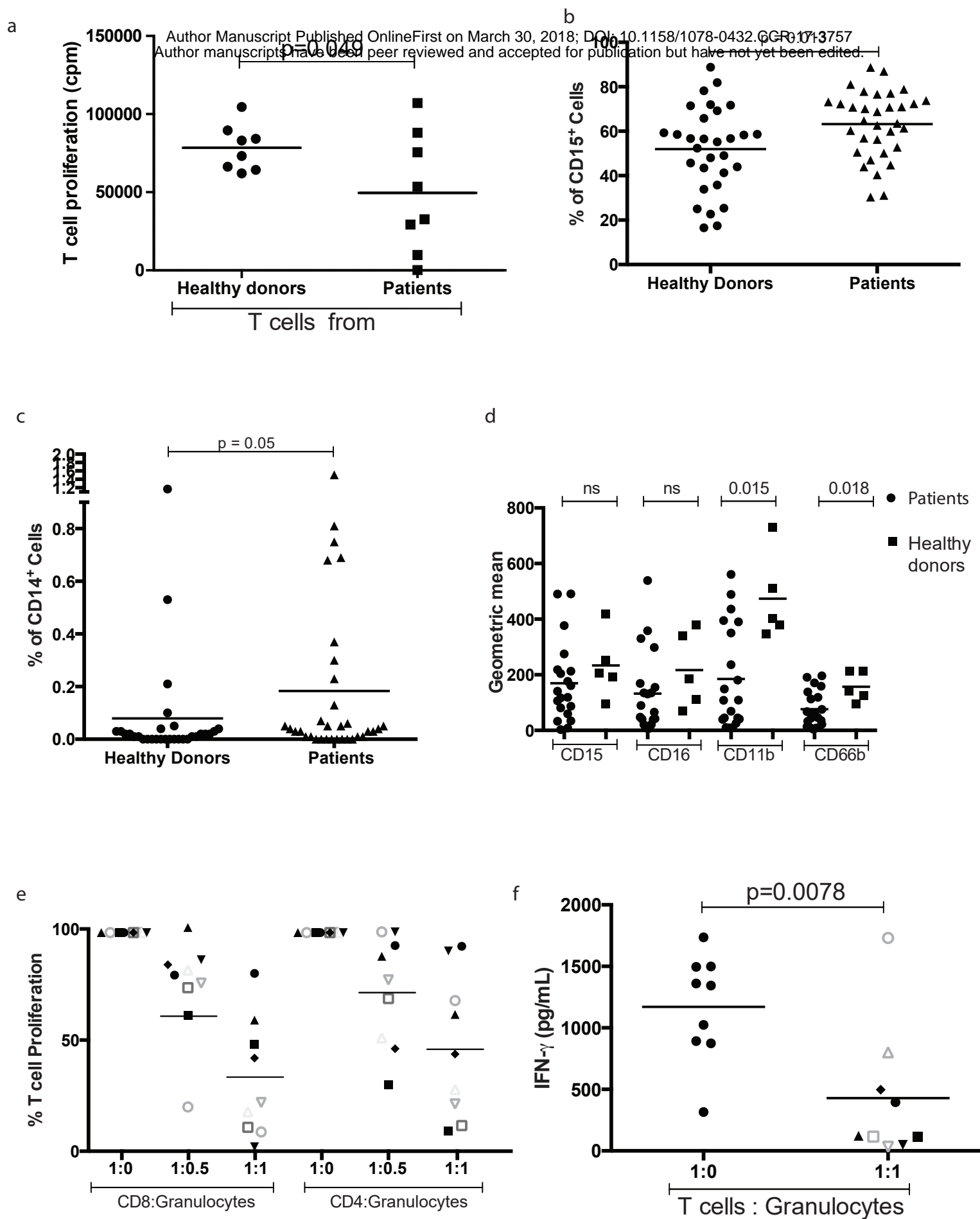
suppressive activity a) Cytokine multiplex assay determined the cytokine profile of tumor cell supernatants and cell line supernatants. Increased concentrations of GM-CSF, IL-8, GCSF, VEGF, IL-6 and mesothelin are found. Low concentrations of prostaglandin E2 and IL-13 were detected. b) Transcriptomic expression of GMCSF, GCSF, IL-6, IL-13, IL-8, VEGF, and mesothelin in 87 mesothelioma tumours from the R2: Genomics and Visualisation Platform c) ROS production (DCFDA staining) by healthy donor CD15⁺ cells treated with detected cytokines to determine which were capable of enhancing ROS production. GM-CSF increased ROS production most prominently. d) T cell proliferation was significantly suppressed by granulocytes conditioned with recombinant GM-CSF, compared to control granulocytes. Ratios of 1:1 and 1:0.5 T cells:granulocytes shown e) Inhibition of granulocyte ROS production (iNAC) or accumulation (catalase) after healthy donor granulocytes were conditioned with GM-CSF, restores T cell proliferation compared to controls.

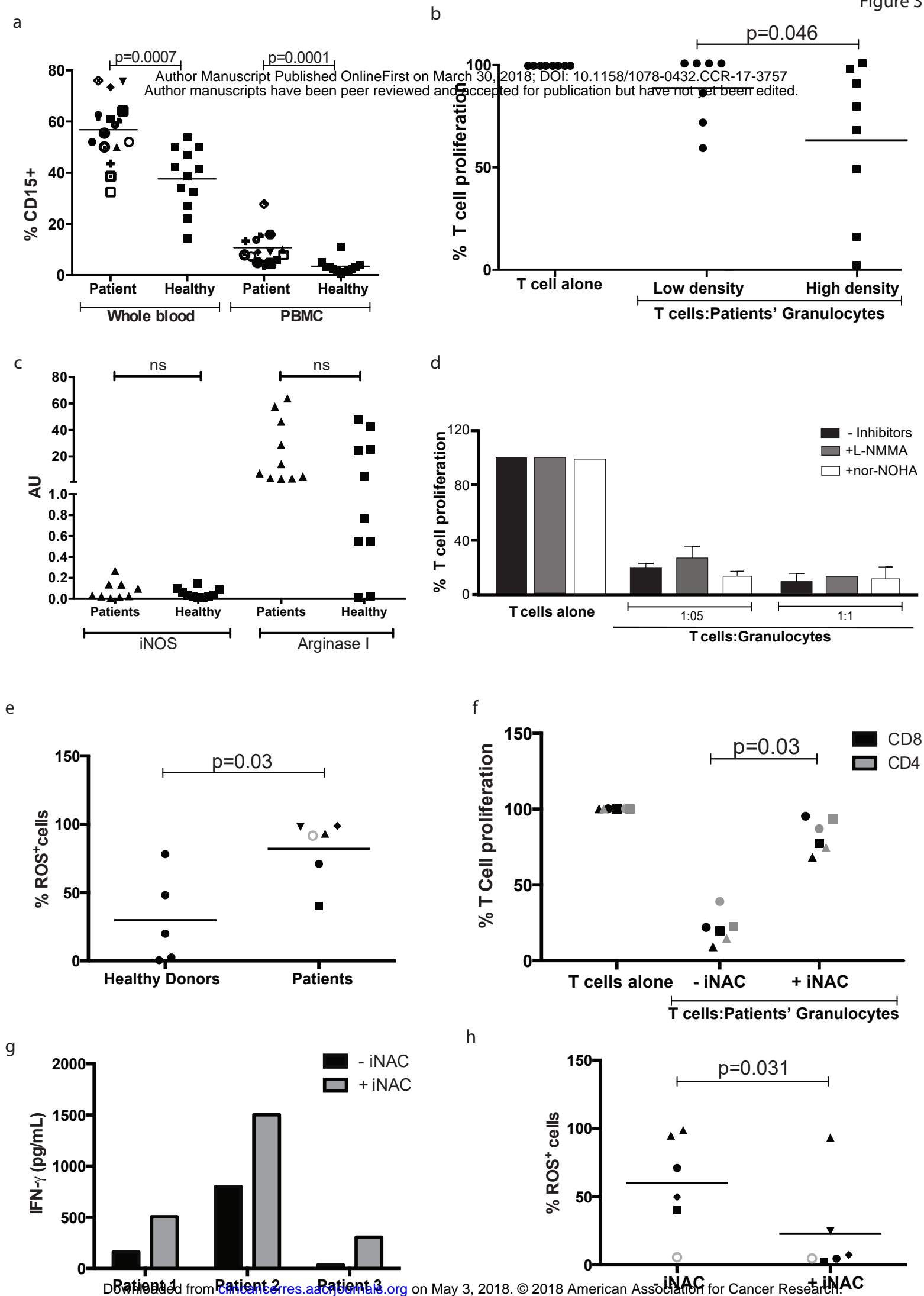
Figure 6: Anti-GM-CSF neutralizing antibody can prevent granulocyte suppressive

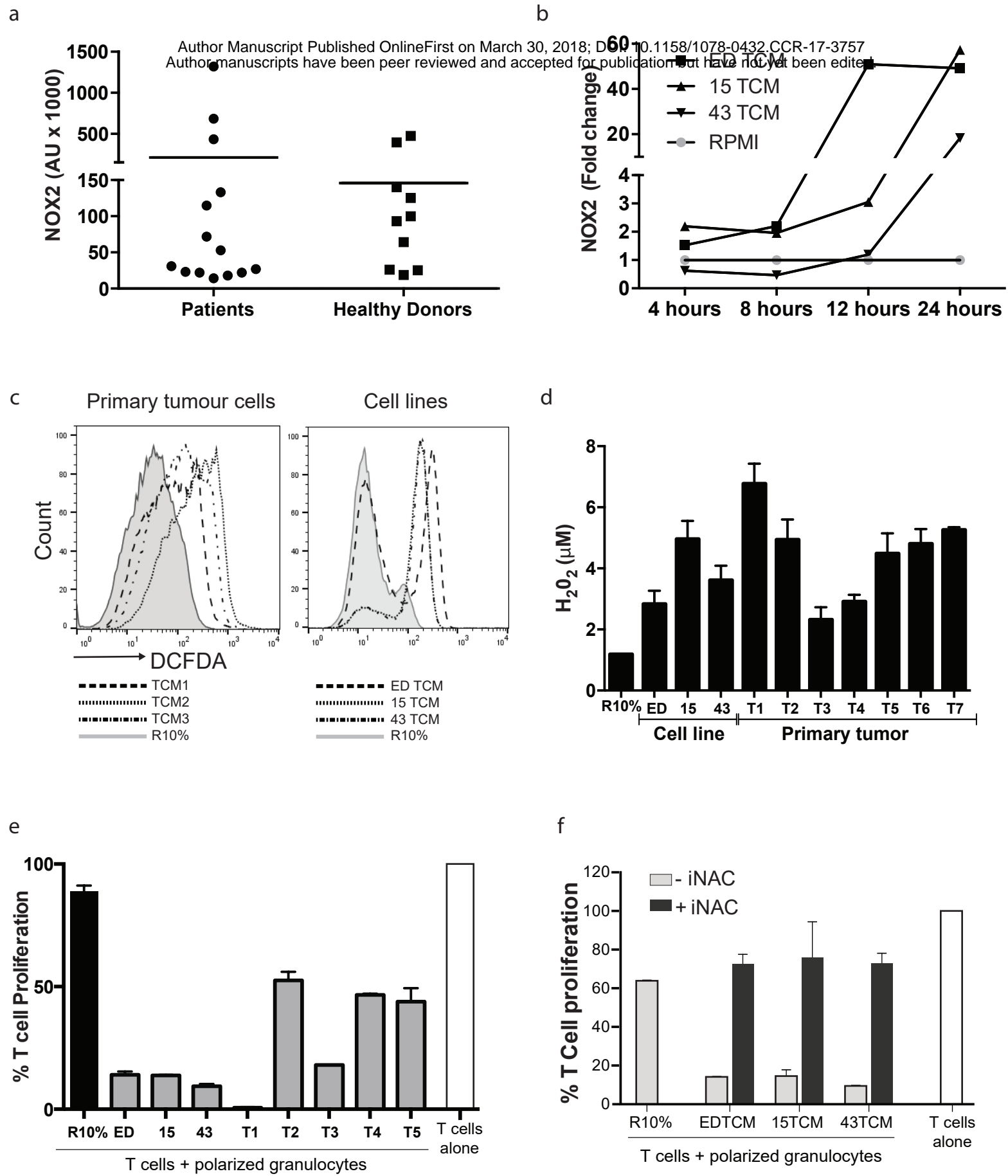
function a) The addition of anti-GM-CSF neutralizing antibody to mesothelioma cell line conditioned media prevents granulocyte ROS expression, as measured by DCFDA staining. Representative histograms for ED cell line shown. b) The addition of anti-GM-CSF neutralizing antibody to mesothelioma cell line conditioned media prevents granulocyte ROS expression. Geometric means for DCFDA staining shown following flow cytometric detection. c) The addition of anti-GM-CSF neutralizing antibody to mesothelioma cell line conditioned media, prevents granulocyte suppressive activity

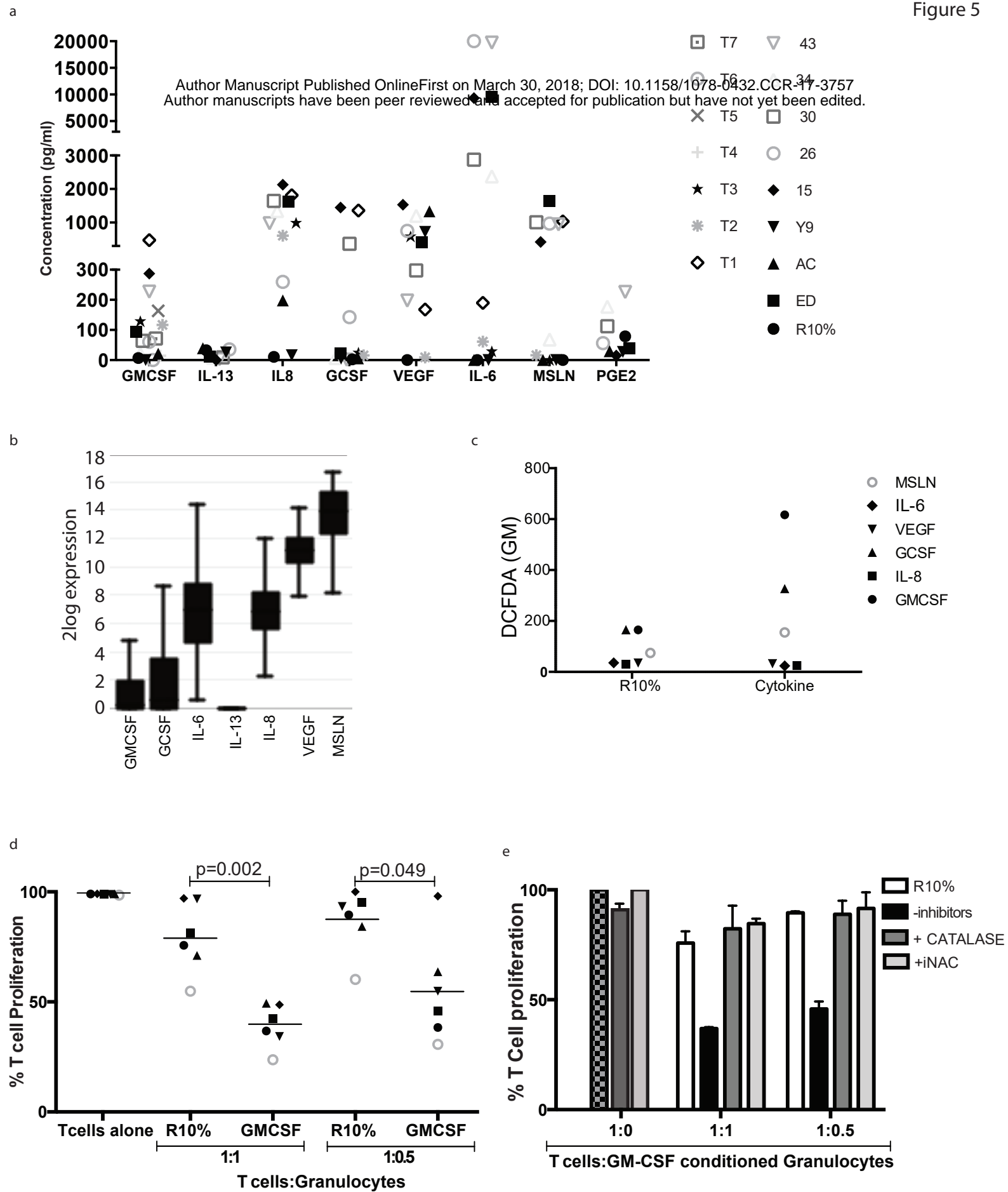
774 compared to granulocytes cultured in tumour-conditioned media alone. d) Correlation
 775 between GM-CSF concentrations in tumour conditioned media and expression of
 776 Reactive Oxygen Species in conditioned granulocytes, as measured by DCFDA mean
 777 fluorescence intensity by flow cytometry. Linear correlation line shown. e)
 778 Immunohistochemistry staining demonstrating the presence of GM-CSF in mesothelioma
 779 tumor sections. Mesothelioma of epithelioid (first image), adenomatoid (second and third
 780 images) and mixed/biphasic (fourth image) type demonstrated cytoplasmic positivity of
 781 tumor cells in a diffuse pattern. Images taken at 20 X magnification. (f) Interrogation of
 782 87 primary tumor samples within the R2: database demonstrated that GM-CSF is
 783 expressed in over 50 % of the samples, and did not correlate with histological subtype.

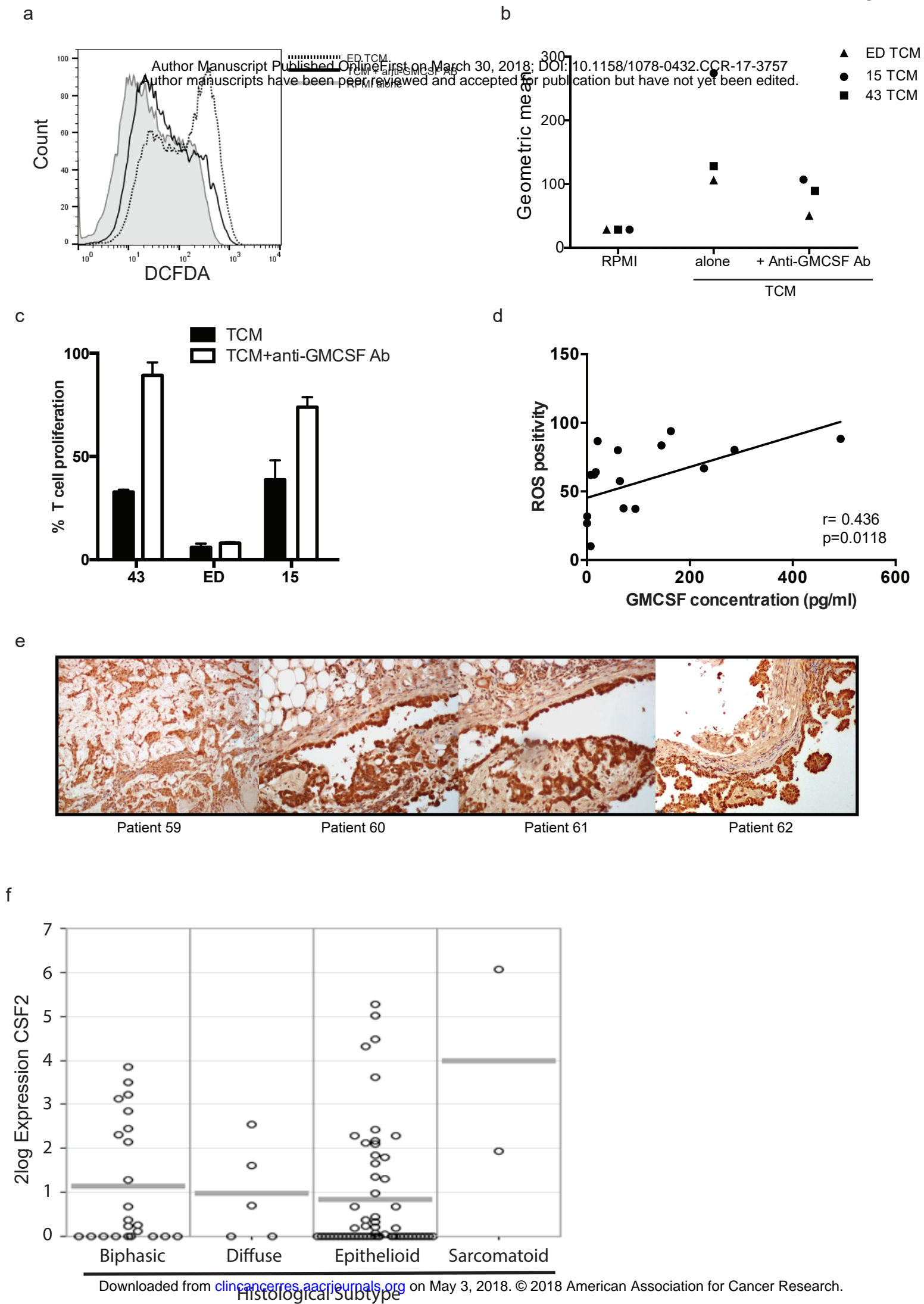












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Tumour-derived GM-CSF promotes granulocyte immunosuppression in mesothelioma patients

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